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User Manual

Cignal Finder™ Multi-Pathway Reporter Arrays (Plate Format)

Cell-Based Multi-Pathway Activity Assays

Part # 1036A Version 1.5 1/10/2011

SABiosciences Corporation 6951 Executive Way, Frederick, MD 21703 USA

Cignal Finder™ Multi-Pathway Reporter Arrays (plate format)

Cell-Based Multi-Pathway Activity Assays

User Manual

(For Catalog Numbers CCA-1XXL or CCA-901L)

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I. Introduction

The Cignal Finder Multi-Pathway Reporter Arrays enable you to pinpoint the pathways regulated by the gene products or chemical compounds being studied in your laboratory. The Cignal Finder Arrays consist of 10 or 45 dual-luciferase reporter assays, and are designed for use in one of four research areas. The targeted research areas are cancer, immunology, development, and toxicology. In this era of post-genomics life science research, many labs are investigating how diverse signal transduction pathways function on their own, and in combination, within the cell. The Cignal Finder Arrays equip life science researchers to carry out such studies with speed and confidence.

These arrays are cell culture-ready 96-well plates. For the 10-pathway arrays, each of the twelve columns of the 96-well plate contains a pathway-focused reporter or control dried down in all eight wells. For the 45-pathway array, each pathway reporter assay is dried down in two wells, with the remaining wells being used for positive and negative controls. The reporter assays are reverse transfected into your cells.

Each pathway-focused dual-luciferase reporter encodes for the mammalian codon-optimized, non-secreted form of the firefly luciferase gene, carrying a protein-destabilizing sequence. Cells rapidly degrade the destabilized form of the firefly luciferase protein and hence the background luciferase activity (noise level) is greatly reduced. Due to low background activity, the magnitude of the response that can be measured (signal-to-noise ratio) as well as the speed of measuring changes in transcription are enhanced. The Cignal dual-luciferase reporter assays provide outstanding reproducibility, sensitivity, specificity, and signal-to-noise ratio. The Cignal reporters are useful assays for carrying out quantitative pathway regulation studies.

Benefits of Cignal Finder Multi-Pathway Reporter Arrays

- **Multi-Pathway Analysis**: Profile the changes in the activities of ten or forty-five signaling pathways relevant to a specific biological process

- **High Performance**: Dual-luciferase assay provides high sensitivity, specificity, and reproducibility

- **Flexibility and Convenience**: Utilize a straightforward reverse transfection procedure with your favorite cell lines to rapidly generate valuable mechanism of action data

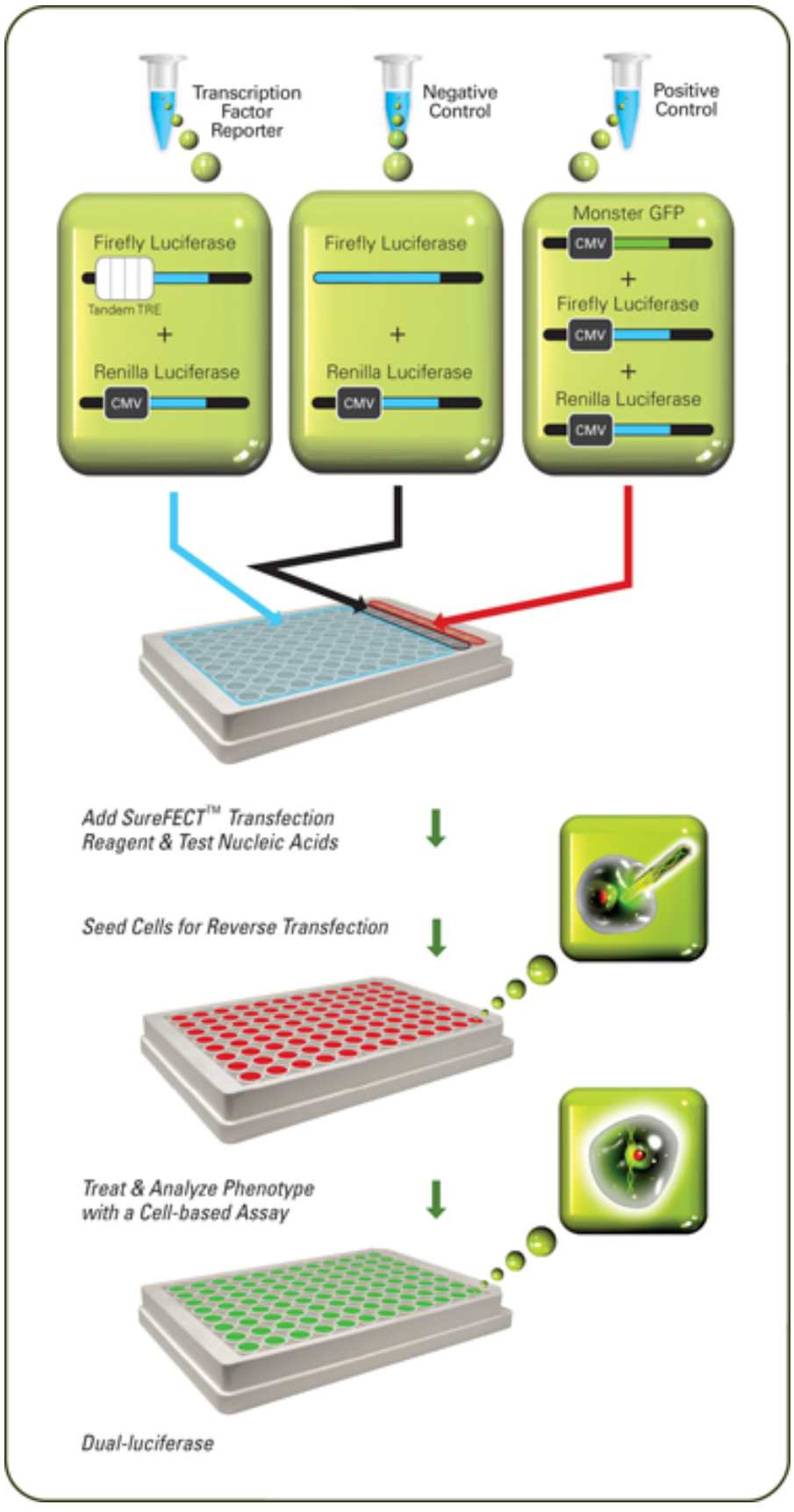


Figure 1: Overview of Cignal Finder™ 10-Pathway Reporter Array Protocol.

II. Product Contents and Descriptions

A. Product Contents

Cignal Finder 10-Pathway Reporter Array Contents:

Table 1: Cignal Finder Reporter Array (plate format) Specifications

Component	Specification	Total DNA in each well
Each of the 10 Reporter Assays	A mixture of an inducible transcription factor responsive firefly luciferase reporter and constitutively expressing *Renilla* construct (40:1).	200 ng
Negative control	A mixture of non-inducible firefly luciferase reporter and constitutively expressing *Renilla* construct (40:1).	200 ng
Positive control	A mixture of a constitutively expressing GFP construct, constitutively expressing firefly luciferase construct, and constitutively expressing *Renilla* luciferase construct (40:1:1).	200 ng

Cignal Finder 45-Pathway Reporter Array Contents:

Table 2: Cignal Finder Reporter Array (plate format) Specifications

Component	Specification	Total DNA in each well
Each of the 45 Reporter Assays	A mixture of an inducible transcription factor responsive firefly luciferase reporter and constitutively expressing *Renilla* construct (40:1).	200 ng
Negative control	A mixture of non-inducible firefly luciferase reporter and constitutively expressing *Renilla* construct (40:1).	200 ng
Positive control	A mixture of a constitutively expressing GFP construct, constitutively expressing firefly luciferase construct, and constitutively expressing *Renilla* luciferase construct (40:1:1).	200 ng

NOTE: *All constructs are **transfection-grade** and are ready for transient transfection. These constructs are specifically designed to inhibit transformation and are NOT MEANT for introduction and amplification in bacteria.*

Each kit also includes a **white self-adhesive sealing tape** for each plate included in the kit. This tape should be affixed to the bottom of each plate immediately prior to reading the plate in a plate-reading luminometer, in order to maximize the signal-to-noise ratio of each reading.

B. Description of Individual Cignal Reporter Assays:

Each Cignal Reporter Assay Kit includes the following components:

1. **Reporter:** Each reporter is a mixture of an inducible transcription factor responsive construct and constitutively expressing *Renilla* luciferase construct (40:1). The inducible transcription factor-responsive construct encodes the firefly luciferase reporter gene under the control of a basal promoter element (TATA box) joined to tandem repeats of a specific Transcriptional Response Element (TRE; Figure 2A). This construct monitors both increases and decreases in the activity of a key transcription factor, which is a downstream target of a specific signaling pathway. The constitutively expressing *Renilla* construct encodes the *Renilla* luciferase reporter gene under the control of a CMV immediate early enhancer/promoter (Figure 2B) and acts as an internal control for normalizing transfection efficiencies and monitoring cell viability. It is also useful to confirm transfection and to verify active luciferase in the transfected culture.

2. **Negative control:** The negative control is a mixture of non-inducible reporter construct and constitutively expressing *Renilla* luciferase construct (40:1). The non-inducible reporter construct encodes firefly luciferase under the control of a basal promoter element (TATA box), without any additional transcriptional response elements (Figure 2C). The negative control is critical to identifying specific effects and determining background reporter activity.

3. **Positive control:** The positive control is a constitutively expressing GFP construct (Figure 2D), pre-mixed with a constitutively expressing firefly luciferase construct (Figure 2E), and a constitutively expressing *Renilla* luciferase construct (Figure 2B) (40:1:1). The positive control is necessary for visual confirmation of transfection. It is also useful for transfection optimization studies. The expression of the GFP from the positive control construct can be monitored by fluorescence microscopy using an excitation filter of 470 ± 20 nm (470 / 40 nm) and an emission filter of 515 nm (long pass).

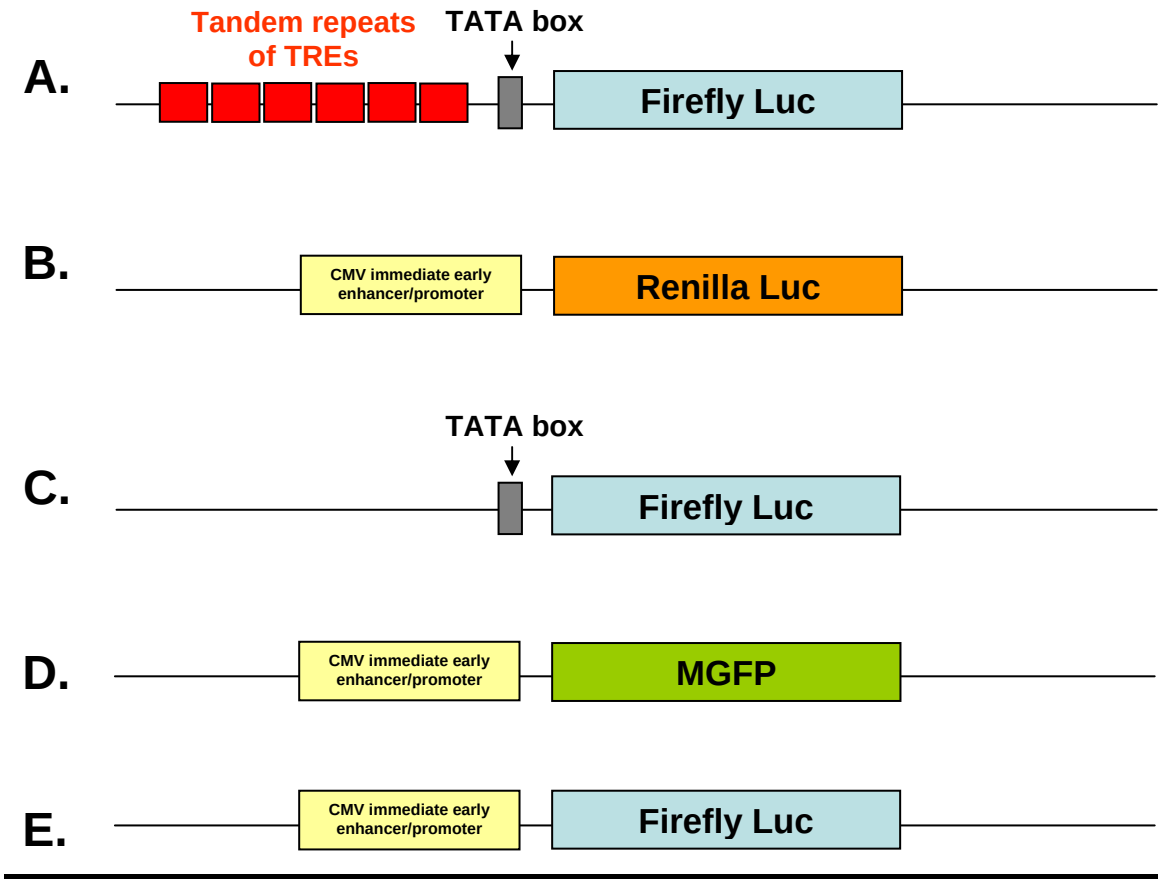


Figure 2: Schematic representation of constructs involved in the Cignal Reporter Assay. (A) The inducible transcription factor-responsive construct expressing firefly luciferase, (B) The constitutively expressing *Renilla* luciferase construct, (C) The non-inducible firefly luciferase reporter construct, (D) The constitutively expressing GFP construct, and (E) The constitutively expressing firefly luciferase construct.

IMPORTANT NOTE: There are a few reports in the literature of the CMV regulatory element being activated by certain stimuli (see below). SABIosciences recommends that you confirm that the stimulus used in each Cignal reporter assay does not induce the CMV regulatory element, in order to confirm that the CMV-Renilla construct is the appropriate normalization construct for the experiment. This can be done empirically by testing the impact of a stimulus on the Cignal positive control reporters, which are each under the control of the CMV enhancer/promoter cassette. If stimulus is one of the very few reported activators of the CMV regulatory element, SABiosciences advises contacting technical support.

- W. Bruening, B. Giasson, W. Mushynski, and H. D. Durham. 1998. Nucleic Acids Research 26(2):486-489. **Activation of stress-activated MAP protein kinases up-regulates expression of transgenes driven by the cytomegalovirus immediate/early promoter**

- Madhu S. Malo, Moushumi Mozumder, Alexander Chen, Golam Mostafa, Xiao Bo Zhang, Richard A. Hodin. 2006. Analytical Biochemistry 350:307-309. **pFRL7: An ideal vector for eukaryotic promoter analysis**

III. Additional Materials Required:

- Mammalian cell line cultured in the appropriate growth medium
- Cell culture medium and standard cell culture supplies
- Multi-channel pipettor and pipettor reservoirs
- Transfection reagent [Recommended reagent: SureFECT Transfection Reagent (SABiosciences, Cat. No. SA-01), however, other transfection reagents work equally well]
- Polystyrene test tubes (BD FALCON, Cat # 352099)
- Opti-MEM® I Reduced Serum Medium (Invitrogen, Cat. No. 31985-062)
- Fetal bovine serum (FBS)
- Non-essential amino acids (NEAA) (Invitrogen, Cat. No. 11140-050)
- Penicillin/Streptomycin
- Hemacytometer
- Dual-Luciferase® Assay System
    - Dual-Luciferase® Reporter Assay System (Promega, Cat. No. E1910)
      This system requires cell lysis, and is well-suited for the rapid quantitation of both luciferase reporters when using luminometers with reagent auto-injectors.
    - Dual-Glo® Luciferase Assay System (Promega, Cat. No. E2920)
      This system is used to assay for both luciferase reporters on intact cells in growth medium. This system can be used with any luminometer, including those without reagent auto-injectors.
- 96-well white opaque flat bottom microtiter plate
- Luminometer

IV. Protocol:

A. Before you begin:

1. Cell line selection: The Cignal Reporter Assay may be used with various mammalian cell lines. Cell lines show a great deal of variation in the levels of signaling proteins. The transcriptional activator activities in the cell line used will determine the sensitivity of the assay. A cell line should be selected based on the functionality of the signal transduction pathway under investigation, as well as for the "transfectability" of the cell line (see below).

2. Transfection reagent selection: SABiosciences recommends the use of SureFECT (SABiosciences, Cat. No. SA-01) as a transfection reagent. The Cignal Reporter Assay, however, also performs equally well with other transfection reagents. When using alternative transfection reagents, please refer to the manufacturer's instructions on the use of those reagents.

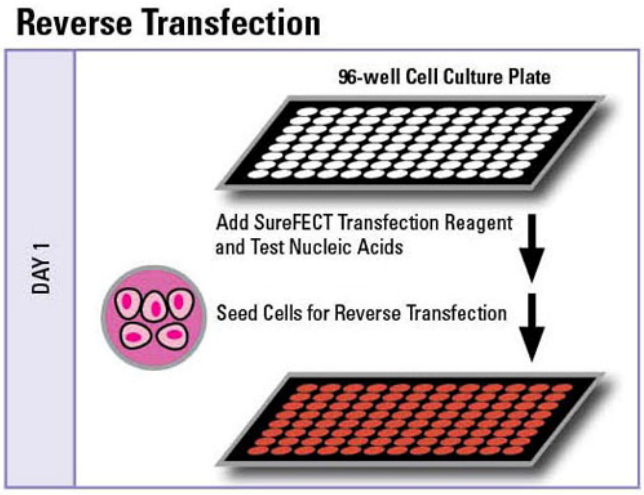
3. Optimization of transfection conditions: The sensitivity of the Cignal Reporter Assay depends on the transfection efficiency. The transfection efficiency, in turn, primarily depends upon cell line used. Therefore, it is very important to optimize the transfection conditions for each cell type under study. Variables to consider, when optimizing the transfection conditions include cell density, cell viability, amount of DNA, ratio of DNA to transfection reagent, transfection complex formation time, and transfection incubation time (see the detailed protocols for our recommendations). The positive control construct included with each Cignal Reporter Assay can be used for determining the optimal transfection conditions.

4. Optimization of assay condition: The response rate in the Cignal Reporter Assay depends on the assay conditions (conditions of the experimental treatment). To obtain maximum response given by any stimulus, perform dosing and time-course studies. The optimal amount of stimulus and the time of treatment must be obtained empirically for each experiment (see different protocols for our recommendations).

5. Important recommendations for best results:
    A. Perform all transfections in **triplicate** to minimize variability among treatment groups.
    B. Include positive and negative controls in each experiment to obtain reliable results.
    C. Use low-passage cells that are actively growing and are greater than 90% viable, for maximal transfection efficiencies.
    D. Do not add antibiotics to media during transfection, as this may cause cell death.
    E. Take care to always seed the same number of cells in each well, in order to maximize the reproducibility of your experiment.
    F. Serum induces various signaling pathways, leading to cross-talk and high background. Therefore, use reduced amounts of serum (0.5%) in the assay medium during the experimental treatment to minimize these serum effects.

6. Transfection Protocols: In order to use the Cignal Finder 10 or 45-Pathway Arrays in the plate format, a reverse transfection method must be employed. This approach involves seeding the cell line of interest onto the transfection complexes in a one day procedure. This is in contrast to traditional transfection methods, in which cells are seeded on the first day of the experiment and transfection complexes are added to the cells the following day. The SureFECT transfection reagent has been specifically developed as a reverse transfection reagent. Optimized reverse transfection protocols using the SureFECT transfection reagent are described throughout the Cignal Finder Reporter Arrays User Manual. Utilizing reverse transfection procedures results in both a time savings as well as improved reproducibility, when compared to traditional forward transfection methods. Conditions for using transfection reagents from other vendors in reverse transfection protocols may also be developed. This will require initial process optimization studies. Below is a general protocol overview for reverse transfection of the Cignal Finder 10- or 45-Pathway Reporter Arrays.

Reverse Transfection Protocol Overview (1 DAY PROCEDURE)



- Add 50 µL of Opti-MEM to each well of Cignal Finder array plate to resuspend reporter constructs
- Dilute SureFECT into Opti-MEM
- Add 50 µL of diluted SureFECT to 50 µL of resuspended reporter constructs, mix well and incubate at room temperature for 20 minutes
- Trypsinize (if necessary), count, and suspend cells to appropriate density
- Immediately seed 50 µL of suspended cells to each well
- Replace growth medium after 16-24 hours of transfection

B. PROTOCOL

The following protocol is designed to reverse transfect an adherent cell line, HEK293, using SureFECT Transfection Reagent (Cat. No. SA-01). *If you are using a transfection reagent other than SureFECT follow their manufacturer's protocol for optimizing transfection.* **This is just a general guideline; the optimal conditions/amounts should be optimized according to the cell type and the study requirements.** Read the protocol completely before starting the experiment.

IMPORTANT: (1) Do not add antibiotics to media during transfection as this causes cell death. (2) Avoid the use of DMEM medium.

1. Add 50 µl of Opti-MEM® to each well of the Cignal Finder Array plate (avoid using DMEM). Resuspend the reporter assay constructs by gently tapping the side of the plate, while slightly rocking the plate back and forth, then left to right, five times each and incubate it for 5 minutes at room temperature.

2. SABiosciences uses 0.3 µl of SureFECT in 50 µl of Opti-MEM® per well for each individual transfection. In order to prepare sufficient SureFECT for an entire 96-well plate, SABiosciences recommends diluting 32.4 µl of SureFECT into 5400 µl of Opti-MEM® (sufficient for 108 transfections). Mix gently by inverting tube slowly and set the tube at room temperature for 5 minutes.

3. After the 5 minute incubation, add 50 µl of diluted SureFECT into each well containing 50 µl of the diluted nucleic acids (1:1 ratio).

4. Mix by gently tapping the sides of the plate for at least 30 seconds and incubate for 20 minutes at room temperature to allow complex formation to occur.

5. Meanwhile, wash cells in a culture dish once with Dulbecco's PBS **without calcium and magnesium**, and treat with 1-3 ml trypsin-EDTA for 2-5 minutes at 37ºC in a humidified atmosphere containing 5% CO_2. Suspend the cells in 7-9 ml of Opti-MEM® containing 5% of fetal bovine serum, then centrifuge the cells down, remove the supernatant, and resuspend the cells to 6×10^5 cells/ml in Opti-MEM® containing 10% of fetal bovine serum and 1% NEAA*******. To ensure reproducible transfection results, it is important to accurately measure the cell density with a hemacytometer or an automated cytometry device.

6. After the 20 minute incubation for complex formation is completed, mix the cell suspension by several inversions of the tube containing the cells or by gentle pipeting of the cell suspension.

7. Add 50 µl of prepared cell suspension (3×10^4 cells in Opti-MEM® containing 10% of fetal bovine serum) to each well containing constructs-SureFECT complexes. This gives a final volume in each well of 150 µl. Mix gently by rocking the plate back and forth, then left to right. Do not move the plate in a circular motion, as this may cause the cells to preferentially sediment around the edges of each well.

8. Incubate cells at 37°C in a 5% CO_2 incubator for 16-24 hours.

9. After 16-24 hours of transfection, change the medium to complete growth medium (DMEM with 10% FBS, 0.1mM NEAA, 1mM Sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin).

10. Carry out the **luciferase assay** using either the Dual-Luciferase Reporter Assay System or Dual-Glo Luciferase Assay System from Promega. Follow the manufacturer's protocol for developing the assay. Please see specific recommendations in the **Important Notes** section below, for some general recommendations on when to carry out the luciferase assays for different types of studies. Each Cignal Finder Array plate comes along with a white self-adhesive sticker, which should be attached to the bottom of the plate before reading the luciferase activity. Using the sticker to cover the optical bottom of the 96-well plate helps to maximize the signal-to-noise ratio of each reading.

Important Notes: Listed below are general recommendations for different experimental designs.

1. To determine the **effect of siRNA/shRNA** on different cell signaling pathways, we recommend doing transient co-transfection of siRNA/shRNA and reporter constructs. For this one can add 2 pmol of siRNA or 200 ng of shRNA plasmid to the resuspended reporter construct in *step 1* of the protocol. The luciferase assay can be developed 48-72 hours after the co-transfection. Please remember to include negative control siRNA/shRNA to assist in the interpretation of your results.

2. To determine the **effect of cDNA overexpression** on different cell signaling pathways, we recommend doing the transient co-transfection of experimental vector and reporter constructs. For this one can add 100-200 ng of experimental vector to the resuspended reporter construct in *step 1* of the protocol. The luciferase assay can be developed 36-48 hours after the co-transfection. Please remember to include negative control vector(empty vector) to assist in the interpretation of your results.

3. To determine the **effect of recombinant protein or small peptide** on different cell signaling pathways, we recommend changing the cell medium to assay medium (Opti-MEM® containing 0.5% of fetal bovine serum, 1% NEAA, 100 U/ml Penicillin and 100 µg/ml Streptomycin) instead of growth medium in *step 9* and treating the transfected cells with 3 or 4 different concentrations of recombinant protein or small peptide 6 to 24 hours prior to assay development.

4. To determine the **effect of small chemicals** on different cell signaling pathways, we recommend changing the cell medium to assay medium (Opti-MEM® containing 0.5% of fetal bovine serum, 1% NEAA, 100 U/ml Penicillin and 100 µg/ml Streptomycin) instead of growth medium in *step 9* and treating the transfected cells with 3 or 4 different concentrations of small chemicals 6 to 24 hours prior to assay development.

For any other troubleshooting or technical questions about the Cignal Reporter Assay, please call one of our Technical Support representatives at 1-888-503-3187 or 301-682-9200 or email at support@SABiosciences.com.

Appendix:

Cignal Finder 10-Pathway Reporter Arrays

Cignal Finder Cancer 10-Pathway Reporter Array

(Tube Format: CCA-001L; Plate Format: CCA-101L)

	Transcriptional Regulatory Element (TRE)	
Pathway		**Transcription Factor**
Wnt	TCF/LEF response element	TCF/LEF
Notch	RBP-Jκ binding element	RBP-Jκ
p53/DNA Damage	p53 response element	p53
TGFβ	SMAD response element	SMAD2/SMAD3/SMAD4
Cell Cycle/pRb-E2F	E2F binding element	E2F/DP1
NFκB	NFκB binding element	NFκB
Myc/Max	E-box binding element	Myc/Max
Hypoxia	HIF response element	Hypoxia-inducible factor-1 (HIF-1)
MAPK/ERK	Serum response element (SRE)	Elk-1/SRF
MAPK/JNK	AP-1 binding element	AP-1

Cignal Finder Immune Signaling 10-Pathway Reporter Array

(Tube Format: CCA-008L; Plate Format: CCA-108L)

	Transcriptional Regulatory Element (TRE)	
Pathway		**Transcription Factor**
NFκB	NFκB binding element	NFκB
Type I Interferon	Interferon stimulated response element (ISRE)	STAT1/STAT2
Interferon Gamma	Interferon gamma activation sequence (GAS)	STAT1/STAT1
IL-6	STAT3 binding element	STAT3
Interferon Regulation	IRF-1 binding element	IRF-1
TGFβ	SMAD response element	SMAD2/SMAD3/SMAD4
cAMP/PKA	cAMP regulatory element (CRE)	CREB
PKC/Ca^{++}	NFAT response element	NFAT
C/EBP	C/EBP binding element	C/EBP
Glucocorticoid Receptor	Glucocorticoid response element (GRE)	Glucocorticoid Receptor (GR)

Cignal Finder Development 10-Pathway Reporter Array

(Tube Format: CCA-003L; Plate Format: CCA-103L)

	Transcriptional Regulatory Element (TRE)	
Pathway		**Transcription Factor**
Notch	RBP-Jκ binding element	RBP-Jκ
Wnt	TCF/LEF response element	TCF/LEF
Myc/Max	E-box binding element	Myc/Max
NFκB	NFκB binding element	NFκB
TGFβ	SMAD response element	SMAD2/SMAD3/SMAD4
Cell Cycle/pRb-E2F	E2F binding element	E2F/DP1
C/EBP	C/EBP binding element	C/EBP
cAMP/PKA	cAMP regulatory element (CRE)	CREB
MAPK/ERK	Serum response element (SRE)	Elk-1/SRF
MAPK/JNK	AP-1 binding element	AP-1

Cignal Finder Stem Cell & Differentiation 10-Pathway Reporter Array

(Tube Format: CCA-006L; Plate Format: CCA-106L)

Pathway	Transcriptional Regulatory Element (TRE)	Transcription Factor
Oct4	Oct4 binding element	Oct4
Nanog	Nanog binding element	Nanog
KLF4	KLF4 binding element	KLF4
Sox2	Sox2 binding element	Sox2
Myc/Max	E-box binding element	Myc/Max
Hedgehog	Gli binding element	Gli
Notch	RBP-Jκ binding element	RBP-Jk
Wnt	TCF/LEF response element	TCF/LEF
Pax6	Pax6 binding element	Pax6
MEF2	MEF2 binding element	MEF2

Cignal Finder Nuclear Receptors 10-Pathway Reporter Array

(Tube Format: CCA-005L; Plate Format: CCA-105L)

Pathway	Transcriptional Regulatory Element (TRE)	Transcription Factor
Estrogen	Estrogen Response Element	Estrogen Receptor (ER)
Androgen	Androgen Response Element	Androgen Receptor (AR)
PPAR	PPARbinding element	PPAR
Retinoic Acid	Retinoic Acid Response Element	RAR
Vitamin D	Vitamin D Response Element	Vitamin D Receptor (VDR)
Glucocorticoid	Glucocorticoid Response Element	Glucocorticoid Receptor (GR)
Progesterone	Progesterone Response Element	Progesterone Receptor (PR)
Retinoid X	RXR binding element	RXR
Liver X	LXR binding element	LXRa
Hepatocyte Nuclear Factor 4	HNF4 binding element	HNF4

Cignal Finder Stress & Toxicity 10-Pathway Reporter Array

(Tube Format: CCA-007L; Plate Format: CCA-107L)

Pathway	Transcriptional Regulatory Element (TRE)	Transcription Factor
Antioxidant Response	Antioxidant Response Element (ARE)	Nrf2/Nrf1
DNA Damage	p53 response element	p53
NFκB	NFκB binding element	NFκB
Hypoxia	HIF response element	HIF-1a
ER Stress	ER Stress Response Element (ERSE)	CBF/NF-Y/YY1
Heavy Metal Stress	MTF1 binding element	MTF1
Heat Shock	Heat Shock Response Element (HSE)	HSF-1
Glucocorticoid	Glucocorticoid response element (GRE)	Glucocorticoid Receptor (GR)
MAPK/JNK	AP-1 binding element	AP-1
Xenobiotic	Xenobiotic Response Element	AhR

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Cignal Finder™ Multi-Pathway Reporter Arrays (Plate Format)

Part #1036A Version 1.5 1/10/2011

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